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TITLE: **Rapid Isolation and Detection for RNA Biomarkers for TBI Diagnostics**

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14. ABSTRACT

Our project work is focused on using a new dielectrophoresis (DEP) microarray technology for rapid isolation and detection of brain-specific RNA and protein biomarkers for TBI diagnostics and patient monitoring. Initial year one project goals include developing DEP techniques for isolation of cell-free (cf) RNA from glioblastoma exosomes and TBI samples (*IRB dependent*); methods for on-chip fluorescence detection of exosomes, RNA, and protein biomarkers; methods for isolation of mRNA and miRNA from exosomes; and development of RT-PCR techniques and specific primers for identifying brain-specific mRNAs, miRNAs and protein biomarkers. We have been successful in demonstrating DEP isolation of glioblastoma exosomes from 50 μL of un-diluted plasma in fifteen to twenty minutes. We also showed tri-color fluorescent detection of the isolated exosomes (red fluorescence), ccf-RNA (green fluorescence) and ccf-DNA (blue fluorescence). Glioblastoma-specific EGFRvIII mRNA from exosomes isolated by DEP, as well as more universal beta-actin mRNA biomarkers, were identified by RT-PCR. We have now also developed a novel on-chip method to carryout immunofluorescence analysis of brain specific exosomal protein biomarkers. We believe this new technique is very close to achieving true Sample to Answer diagnostics.

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1. INTRODUCTION

Current limitations for detection and accurate diagnosis of Traumatic Brain Injury (TBI) in point-of-care (POC) settings pose an ongoing capability gap. For soldiers in training and combat, rapid diagnosis and monitoring of mild, moderate or repeated TBI is of absolute importance. Any brain trauma in the field may not only have serious short-term implications, but also may progress to chronic and debilitating long-term physiological and psychological problems for soldiers and veterans. Our project work is focused on using a new dielectrophoresis (DEP) microarray technology for rapid isolation and detection of brain-specific RNA and other biomarkers for TBI diagnostics and patient monitoring. Original (Year One) project goals included developing the basic DEP techniques for isolation of brain exosomes (glioblastoma cell model) containing cell-free (cf) RNA and protein biomarkers directly from blood, plasma, serum or CSF; methods for isolation of specific mRNA, miRNA and proteins from the isolated glioblastoma exosomes; initial development of specific primers, probes, RT-PCR and other techniques for identifying brain-specific RNA and protein biomarkers; and novel methods for rapid on-chip or in-situ fluorescence detection of the DEP isolated exosomes, RNA and protein biomarkers. Original plans for second phase (Year Two) included further development and improvements of the basic DEP techniques for isolation of exosomes and associated RNA and protein biomarkers from blood, plasma and serum; further optimization of methods for isolation of specific mRNA, miRNA and proteins from the isolated exosomes; further development of specific primers and RT-PCR techniques; further improvement of on-chip fluorescence detection of exosomal RNA and proteins; and development of an algorithmic approach for correlating RNA detections with TBI severity. The final project (Year Two) goals also included demonstrating DEP isolation and RT-PCR detection of specific RNA and protein biomarkers from TBI patient samples; determining which RNA and protein biomarkers are most used for differentiating types of brain damage; and demonstrating on-chip (DEP device) identification of RNA, protein and other biomarkers. Completion of the project objectives would position the DEP technology for development of a fully integrated, robust, portable sample-to-answer POC system for TBI diagnostics and patient monitoring.

To date, considerable technical progress has been made on demonstrating the proficiency of the DEP technology for isolating brain related exosomes and their associated RNA and protein biomarkers from our glioblastoma brain cell culture model (brain exosomes spiked into plasma). This included demonstration of generic fluorescence detection of RNA and exosomes, and detection of specific brain related mRNA by RT-PCR and specific proteins by rapid on-chip immunofluorescence analysis. Unfortunately, because final approval for our project IRB was not received until June 2016, and a three-month set-up time was necessary for acquiring TBI patient samples it was not been possible to use actual TBI samples to date. However, we now have an approved one-year no-cost project extension and everything is in place to collect TBI patient samples from the UCSD trauma centers.

2. **KEYWORDS**

Traumatic brain injury, TBI, brain-specific, biomarkers, RNA, proteins, exosomes, extracellular vesicles, EVs, dielectrophoresis, DEP, molecular diagnostics, sample-to-answer, point-of-care, PCR, immunofluorescence.

3. ACCOMPLISHMENTS

8th Quarter Accomplishments

Again, it is important to point out that we have received approval for a no-cost project extension (EWOF) from October 1, 2016 to September 30, 2017. Thus, we can continue to carry out further work in order to achieve our final TBI project goals, which required use of TBI patient samples.

With regard to obtaining TBI patient samples for the remaining project work, our CoPI Dr. Clark Chen (MD, PhD, UCSD Moores Cancer Center) now has everything in place to begin collecting TBI samples from UCSD trauma centers. These samples will be collected and processed through Dr. Sharmeela Kaushal (Biorepository Manager, UCSD Moores Cancer Center) after which we will pick them up for our TBI-related RNA and protein biomarker testing and analysis. Additionally, on August 30, 2016 we had an initial meeting with Dr. Josh L. Duckworth M.D., CDR MC USN (Assistant Professor, Department of Neurology, F. Edward Hébert School of Medicine, USUHS) and his staff members in Bethesda MD to begin discussing collaboration on our TBI projects. Dr. Duckworth is also a CoPI on this project. On October 4, 2016, we hosted a second meeting here at UCSD in San Diego to discuss collaboration with his ongoing (Military TBI study). Attending this meeting were Dr. Josh L. Duckworth, M.D. (USUHS); Dr. Ida Babakhanyan, Ph.D. (Research Neuropsychologist/ Sr. Principal Scientist, Defense and Veterans Brain Injury Center (DVBIC), Contractor, General Dynamics Health Solutions); and Dr. Adriane Stebbins (Raytheon Corp, San Diego). Attending from the Heller group was Dr. Jean M. Lewis, Ph.D. (Assistant Project Scientist); Dr. Augustine Obirieze, M.D., Dr. Sejung Kim, Ph.D. (Post-doctoral Fellow) and Benjamin Sarno (graduate student). Following a discussion and a live demonstration of our DEP system, the various aspects for the military TBI project were discussed. The logistics of sample collection in remote military training locations, and the necessity of collecting samples on short notice according to the military's schedule were discussed, as was the possibility of our providing some laboratory personnel support. Training of the personnel and sample collection may start in November 2017. It was agreed by all that this collaboration will be very productive.

8th Quarter Accomplishments (Research)

In addition to carrying out continuing work on optimization of basic DEP procedures for brain exosome isolation and RT-PCR for detection of specific mRNA, we have made substantial progress in developing rapid on-chip immunofluorescent analysis for brain specific proteins.

To this end, we have continued screening antibodies for the ability to detect brain-associated proteins using our DEP on-chip immunoassay. Our strategy is to identify one or more pairs of protein biomarkers that, when analyzed together, enable discrimination of TBI patient samples from those of normal individuals. Brain-associated proteins of interest include Tau protein, ubiquitin c-terminal hydrolase L1 (UCHL1), and glial fibrillary acidic protein (GFAP), as well as the EGF receptor (EGFR), beta-actin, and other general cell-derived proteins as controls. To screen antibodies, we have tested DEP-immobilized exosomes derived from cultured glioblastoma cells (supplied by Dr. Chen). To perform the on-chip immunoassay, we loaded about 25 ul of exosomes, purified from cultured U87 glioblastoma cell supernatant, at a concentration of approximately 1-10 X 10^9 particles/ml, directly onto the DEP chip. We applied AC voltage to the sample on the DEP chip (15 Khz, 14 Vpp) for 10 minutes. With the AC voltage still on, samples were washed with 200 ul of reduced-salt PBS for 10 minutes. Following the wash, AC current was turned off, and a solution of 5% normal goat or sheep serum in PBS was added for 10 min to block non-specific sites of antibody binding. Some proteins, such as CD63, are transmembrane proteins that are external to the exosome membrane, and thus accessible to antibodies without further processing. However, some proteins, such as TSG101, are thought to be internal to the exosomes, and thus may require a further permeabilation step to become accessible to the antibodies. These may include Tau protein, a microtubule-associated protein, and GFAP, a filament protein found in glial cells. Membranes were permeabilized using 0.1% saponin for 10 minutes. Primary antibodies were incubated for 90 minutes at RT. For directly conjugated antibodies, such as Alexa Fluor GFAP, samples were washed, then visualized and photographed for further analysis. For other antibodies, following the wash, AlexaFluor 594 or 488-conjugated secondary antibodies were incubated for an additional 90 minutes at room temperature (RT). Following the final wash, images were taken with a Nikon microscope equipped for epifluorescence. As shown in Figure 1, using DEP we have successfully isolated CD63-positive material, likely to contain exosomes, in 20 minutes directly from brain cancer patient plasma. This isolated material was also positive for Tau and EGFR.

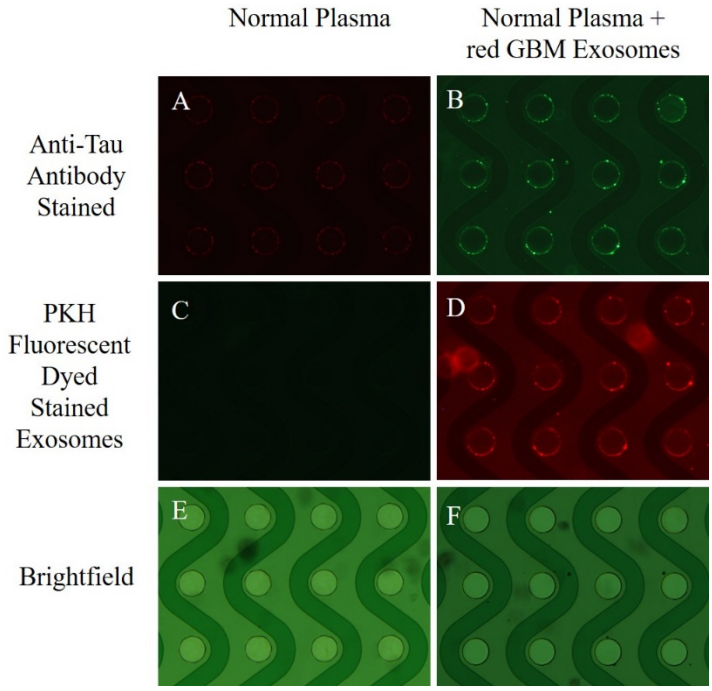


Figure 1 - DEP isolates PKH (red)-dyed glioblastoma exosomes spiked into normal plasma and identified for the brain-specific marker Tau. *Exosomes purified from cultured cell supernatants and stained with the non-specific membrane stain PKH (red) were spiked into normal human plasma. Following isolation onto the DEP chip, exosomes were permeabilized with saponin, and positively labeled with antibody selective for Tau protein. (A and B) Anti-tau antibodies were visualized with Alexa Fluor 488-conjugated secondary antibody (green). (C and D) Fluorescence image of same electrodes, positive for PKH-labeled exosomes (red). (E and F) Electrodes corresponding to panels directly above, viewed with bright field.*

Future Work (9th Quarter and 2017)

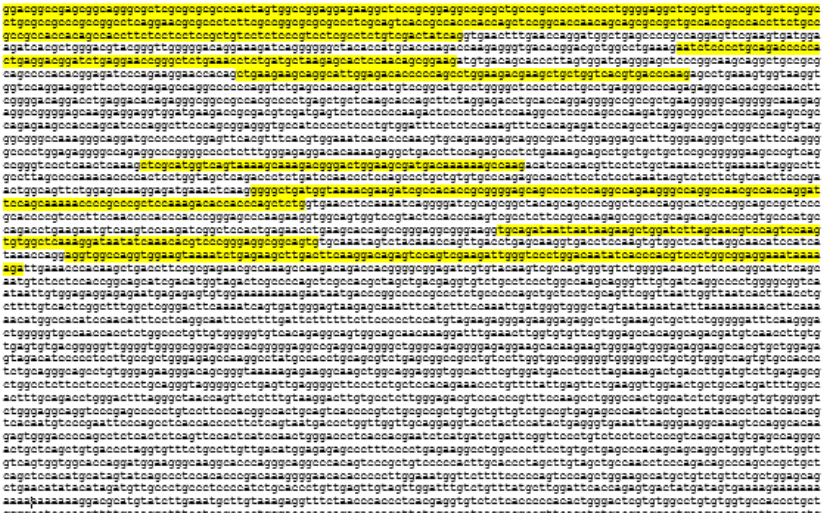
Now that TBI patient samples (blood, plasma, and serum) are available we will begin running these samples, first to show DEP isolation and detection of cf-DNA, RNA and exosomes using generic fluorescent dyes; second to run RT-PCR on mRNA (EGFR, GFAP, tau, UCHL1, PLP, SLC1A, GPM6A, SNAP25), from exosomes isolated by DEP from the TBI samples; and third to demonstrate on-chip immunofluorescence detection of brain-specific proteins from TBI patient samples. We will continue to optimize antibodies for detecting brain-specific proteins that are potential biomarkers for TBI.

RT-PCR Detection of Brain/TBI Specific RNA - We are now prepared to collect and assay TBI samples and continue the work that we have been doing using the glioblastoma exosomes. We will compare our DEP results with the Exiqon kit results to determine the efficacy of our protocol. These results will include images, gel results, qPCR results, and sequencing results when necessary. We will not only assay for beta-actin and EGFR but also other brain-related

mRNA (GFAP, tau, UCHL1, PLP, SLC1A, GPM6A, and SNAP25) and microRNA transcripts. These experiments will help to further improve our DEP protocol.

In addition to the qPCR primers and probes we have obtained for other transcripts of interest, we will design primers for end-point PCR as another way to test for the presence of these transcripts. For this, we need to collect data on sequences, and design unique and robust primers that span introns (to ensure amplification of RNA rather than DNA). One such sequence (tau protein) is shown in Figure 2 below. Each exon is shown by alternating highlighted (yellow) and un-highlighted regions. It can be seen that primers would most likely be designed towards the beginning of the gene in order to keep the amplicon length reasonable. In addition, transcript variants of these genes are often consistent at the beginning and start to vary later in the sequence.

Exon	Start	End
1	1	305
2	306	455
3	456	542
4	543	629
5	630	695
6	696	1448
7	1449	1504
8	1505	1702
9	1703	1829
10	1830	2095
11	2096	2188
12	2189	2270
13	2271	2383
14	2384	6762



We will also continue our quest for downstream on-chip analysis. While we eventually would like to do RT- and qPCR on-chip, the high and rapidly-changing temperatures required for PCR present significant challenges. There are alternatives to traditional PCR that bypass these temperature requirements such as RCA (rolling circle amplification) with padlock probes. RCA is an isothermal reaction that uses strand-displacing polymerases resulting in linear amplification of the padlock probe.

On-Chip Immunfluorescence Detection of Brain/TBI Specific Proteins - In addition to the antibodies already optimized for use in the DEP immunoassay system (such as tau and GFAP), we will also procure and optimize the concentration and incubation times for several additional antibodies. These may include UCHL1, PLP, SLC1A, GPM6A, and SNAP25. As trauma patient samples become available, we will screen each sample in the DEP immunoassay with combinations of the antibodies, collecting fluorescence images for quantitative analysis. Images will be analyzed using ImageJ to quantify, and statistical analysis will be performed to create biomarker profiles for the samples. Initially, we will focus on the biomarkers GFAP and UCHL1, which have been shown recently to have promise as sensitive and/or early markers of TBI (J R Army Med Corps. 2016 Apr;162(2):103-8. doi: 10.1136/jramc-2015-000517. Biomarkers in traumatic brain injury: a review. Toman E). As noted above, our strategy will involve choosing single markers, or combinations of markers, that in our assay can discriminate to some degree the TBI patient samples from those of patients with normal diagnoses.

Figure 2. Exon positions and sequence of Tau protein (MAPT variant 1) transcript (https://www.ncbi.nlm.nih.gov/nuccore/NM_016835.4). Odd exons are highlighted in yellow. Primers that span introns will ensure amplification of RNA rather than DNA.

Some Highlight on Progress, Results and Accomplishments from Earlier Work

In our previous studies, we compared the results of a gold standard RNA isolation kit with our DEP chips. The Exiqon kit was run according to manufacturer instructions and uses 200 uL of sample, which is eluted as 50 uL of purified RNA. After collection of RNA, cDNA is made, and we can assay for a number of biomarkers including beta-actin and GFAP (Figure 3 below).

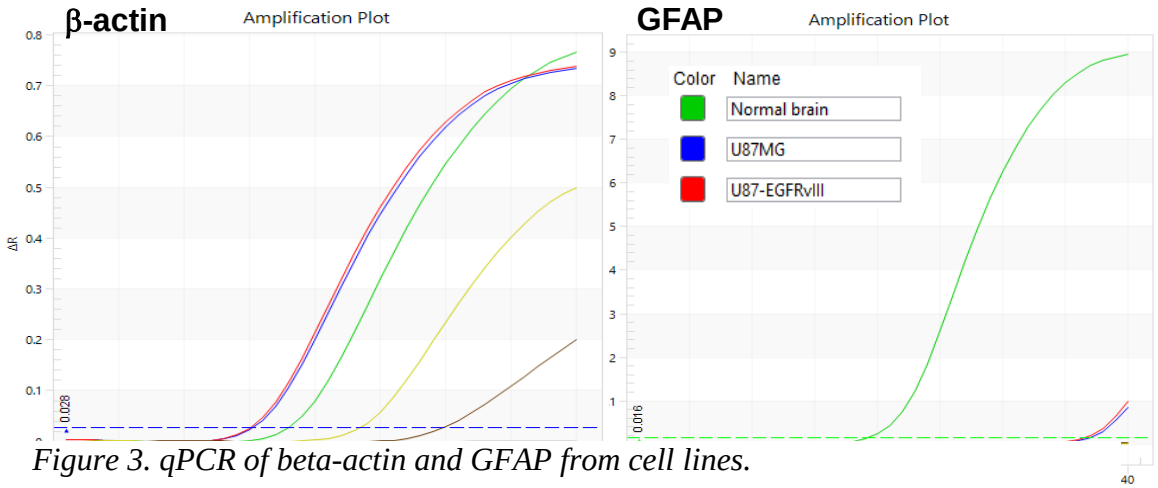


Figure 3. qPCR of beta-actin and GFAP from cell lines.

Our DEP protocol uses about 40 uL of plasma. A voltage of 14 Vpp and frequency of 15 kHz is applied for 10 minutes before washing begins. A wash of 0.5x PBS is run through the chip for 10 minutes at a rate of 20 uL/min. The material collected from the DEP procedure is eluted from the chip (about 35 uL). To visualize collection of DNA, YOYO-1 dsDNA dye is added to the sample at a final concentration of 278 nM before pipetting it onto the chip for DEP. Images are taken after the wash to show fluorescently-labeled capture of DNA. Similarly, to show RNA collection, RNA Select is added to the sample at a final concentration of 2 uM. In addition, we can use fluorescent dyes of different wavelengths to show DEP collection of exosomes and associated RNA in a single image (Figure 4).

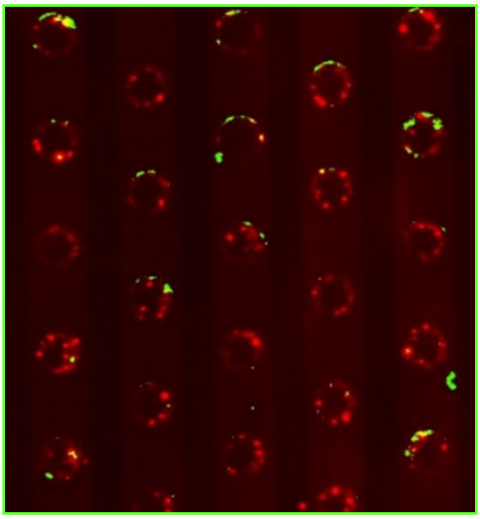


*Figure 4 - **On-Chip Fluorescent Analysis** - Image of U87-EGFRvIII glioblastoma exosomes/EVs that were isolated on the DEP microelectrodes stained with RNA Select green fluorescent dye and the exosomes stained with a PKH membrane specific red fluorescent dye.*

We are currently using the Exiqon kit on DEP-collected samples in order to ensure that the RNA collected is accessible to the reverse transcriptase used to make cDNA. We are closing in on protocols to eliminate this step. We have shown positive results using detergents to permeabilize the exosomes and make the RNA accessible and possible for downstream assaying. Figure 5 shows that we can use saponin in our RT-PCR and subsequent end-point PCR protocols without inhibiting these reactions.

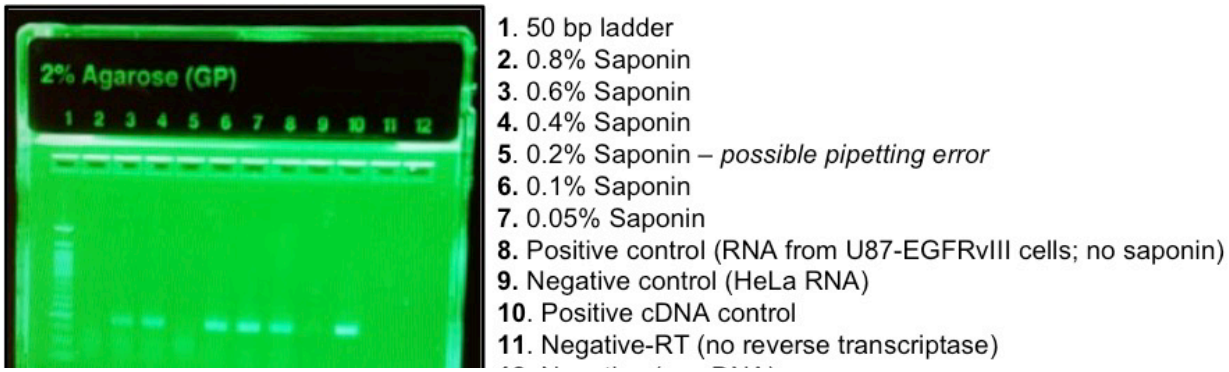


Figure 5 - RT-PCR and subsequent PCR with varying amounts of saponin to break up exosomes and release RNA.

Thus far, we have done extensive studies and protocol optimization using a housekeeping gene, beta-actin. In preparation for work with true TBI samples, we have purchased pre-designed primers and probes for qPCR of more brain-specific mRNA transcripts. These genes include: GFAP, tau protein, and L1cam. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed in mature astrocytes. Tau proteins are proteins that stabilize and provide flexibility to microtubules, mainly in the distal portions of axons. Defective tau proteins have been found to contribute to the pathology of Alzheimer's Disease. Additionally, build-up of abnormal tau has been found in the brains of people with chronic traumatic encephalopathy (CTE). Finally, L1cam is a neuronal cell adhesion molecule that has a role in the development of the nervous system.

We have also purchased pre-designed primers and probes for some micro RNAs that have been shown to be important in characterizing TBI. These are miR-16 and miR-765. Figure 6 shows a preliminary qPCR result for miR-16 using RNA isolated from a glioblastoma cell line.

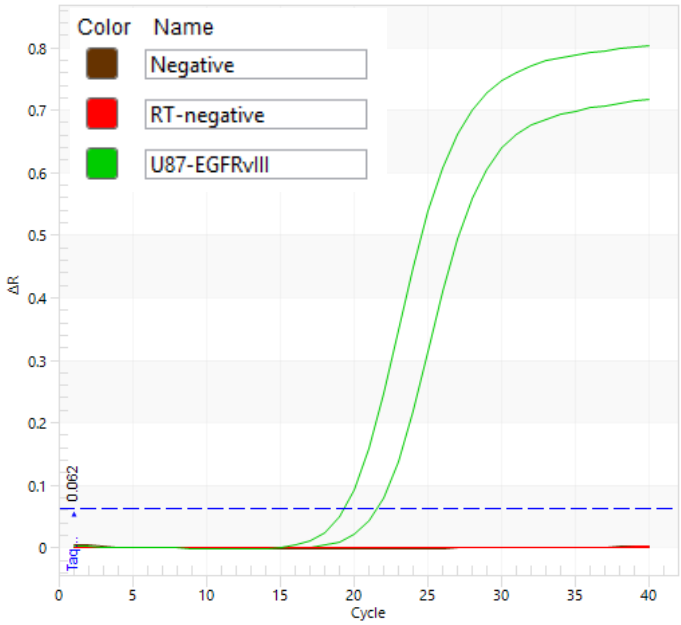


Figure 6 - Amplification of miR-16 TaqMan qPCR of RNA isolated from a glioblastoma cell line.

7th Quarter Accomplishments

Our overall major project accomplishments for year two 7th quarter include:

(1) Received final HRPO approval for our TBI project IRB.

(2) Hired a sample collection person who will be in charge of handling TBI patient samples from the UCSD trauma center hospitals.

(3) Glioblastoma exosomes isolated by DEP from brain cancer patient plasma shown positive by on-chip immunofluorescent detection for CD63, EGFR, and Tau proteins.

(4) Receive approval for no cost project extension (EWOF) from October 1, 2016 to September 30, 2017

(5) Begin the FITBIR registration and data collection process.

Additionally, we have prepared and submitted a manuscript titled ***"Rapid Isolation and Detection of Glioblastoma Exosomes and Associated Protein and RNA Biomarkers from Plasma Using AC Dielectrophoresis"*** to Nature Biotechnology (Now being resubmitted to Nature Nanotechnology)

Also our abstract to the 2016 Military Health System Research Symposium (MHSRS) for the TBI Tract (1) on TBI Diagnostic and Prognostic Indicators which is titled "Isolation and Detection of Exosomes and Associated RNA and Protein Biomarkers for TBI Diagnostics", has been accepted for a poster presentation.

6th Quarter Accomplishments

Our overall major project accomplishments for year one and year two 6th quarter include:

(1) demonstrating that the proposed high conductance DEP (HC-DEP) microarray devices and technology are able to rapidly isolate brain-related (glioblastoma exosome) mRNA and proteins directly from plasma in 15-20 minutes;

(2) showing that the circulating cell-free (ccf) mRNA and other RNAs are encapsulated in vesicles/exosomes which protect it from degradation in the blood/plasma;

(3) demonstrating rapid in-situ/on-chip fluorescence detection of the vesicles/exosomes and associated RNA within 15-20 minutes of sample application to the HC-DEP microarray/chip device;

(4) producing SEM/TEM images of vesicles/exosomes isolated on the HC-DEP microarray/chip surface;

(5) releasing the encapsulated mRNA from the isolated vesicles/exosomes and then identifying the brain-specific mRNA and other mRNA using RT-PCR; and

(6) identifying brain-specific exosome proteins in-situ/on-chip using fluorescent antibodies.

(7) improving techniques for more rapid and efficient DEP isolation and detection of RNA, as well as improved protein detection by on-chip immunofluorescence;

(8) demonstrating new DEP phenomena where large exosome aggregates (> one micron) are collected in the high field regions of the DEP chip; and

(9) showing the presence of Tau protein in U87 glioblastoma exosomes immobilized onto DEP chip by immunofluorescence. All of these were accomplished using the glioblastoma brain cell culture model, for producing exosomes that contain brain-specific RNA and proteins in plasma samples.

5th Quarter Accomplishments

Our overall major project accomplishments for year one and the 5th Quarter include:

(1) demonstrating that the proposed high conductance DEP (HC-DEP) microarray technology is able to rapidly isolate brain related mRNA directly from plasma in 15-20 minutes;

(2) showing that the circulating cell-free (ccf) mRNA and other RNAs are encapsulated in vesicles/exosomes which protect it from degradation in the blood/plasma;

(3) demonstrating rapid in-situ/on-chip fluorescence detection of the vesicles/exosomes and associated RNA within 15-20 minutes of sample application to the HC-DEP microarray/chip device;

(4) producing SEM/TEM images of vesicles/exosomes isolated on the HC-DEP microarray/chip surface;

(5) releasing the encapsulated mRNA from the isolated vesicles/exosomes and then identifying the brain-specific mRNA and other mRNA using RT-PCR; and

(6) now identifying brain specific exosome proteins in-situ/on-chip using fluorescent antibodies. All of these were accomplished using the glioblastoma brain cell culture model, for producing exosomes/RNA in plasma samples.

We feel that it is important to point out that the second year, 5th, 6th, 7th and 8th quarter results clearly demonstrate major project accomplishments that the research literature now also strongly supports: (1) the relationship of cf-RNA with vesicles/exosomes; (2) that for TBI and other CNS diseases/disorders most of the circulating cell-free (ccf) RNA (mRNA, miRNA, etc.) is found encapsulated in vesicles/exosomes/inflammasomes; and (3) the major challenge to using exosomes and associated RNA and proteins as viable biomarkers is the time-consuming, complex and costly procedures now required for their isolation (Nature Reviews/Neurology, Vol 9, June 2013, 331-339; Clinica Chimica Acta 414, 2012, 12-17; Phil. Trans. R. Soc. B 369: 201330502; Phil. Trans. R. Soc. B 369, 20130503; J Neurosurg . 2012 December; 117(6): 1119–1125. doi:10.3171/2012.9.JNS12815; J. of Neurotrauma 27:2147–2156, Dec. 2010; Nature doi:10.1038/nature14581; Clinical Chemistry 62:2 000-000, 2016 – doi/10.1373/clinchem.2015.246538).

4. IMPACT

4.1 Impact on the development of the principal discipline(s) of the project

We believe that our first and second year project work and results will have a significant impact on TBI diagnostics and patient monitoring. We have demonstrated that DEP can be used to rapidly isolate glioblastoma (brain) exosomes, RNA and protein biomarkers directly from plasma. mRNA can be isolated and RT-PCR can be used to detect and identify specific brain-related mRNAs. Additionally, this project has also led to the development of a viable on-chip immunofluorescent method for rapid detection of brain-specific exosomal proteins.

4.2 Impact on other disciplines

The results of first and second year project work on demonstrating rapid DEP isolation of brain-related exosomes, RNA and protein biomarkers from plasma will impact other related areas such as Alzheimer's disease and sports injury. It will also have a significant impact on cancer diagnostics, where isolation of exosomes, RNA, protein biomarker detection is very important for enabling future "liquid biopsy" molecular diagnostics.

4.3 Impact on technology transfer

The results of first and second year project work on demonstrating rapid DEP isolation of brain-related exosomes and cf-RNA from plasma will have impact on technology transfer as it demonstrates overall viability of the DEP technology for a wide variety of diagnostic applications, including liquid biopsy cancer diagnostics. At this time, a new company, Biological Dynamics (San Diego, CA), is in the process of commercializing DEP microarray devices and systems for cancer diagnostics. This is the DEP technology originally developed in the Heller lab and licensed from UCSD by Biological Dynamics. Additionally, along with Raytheon (Project CoPI), we will be evaluating future POC and field packaging for DEP-based diagnostic systems.

4.4 Impact on society beyond science and technology

TBI can lead to other neurodegenerative processes that directly decrease quality of life and have a major impact on the patient's long-term psychological health. This has significant negative effects on family members and is costly to society in general. We believe new TBI molecular diagnostics for patient monitoring and management could identify patients that have had a TBI event allowing earlier medical and therapeutic intervention. This can catch and address complications earlier thereby ameliorating short and long term patient health problems and ultimately reducing the resulting negative effects on family members and society in general.

5. CHANGES/PROBLEMS

5.1 Changes in approach and reasons for change

Because final approval for our project IRB was not received until June 2016 and a three-month set-up time was necessary for acquiring TBI patient samples, it has not been possible to use any actual TBI samples to date. However, since we now have an approved no cost project extension (EWOF) for one year and everything is in place to collect TBI patient samples from the UCSD trauma centers, we can now begin to run TBI samples and complete our project goals.

5.2 Actual or anticipated problems or delays and actions or plans to resolve them

Again, since we now have an approved no cost project extension (EWOF) for one year and everything is in place to collect TBI patient samples from the UCSD trauma centers, we can now begin to run actual TBI samples and complete our project goals.

5.3 Changes that had a significant impact on expenditures

Some adjustments to expenditures were necessary in order to complete project goals during the projects extended period.

5.4 Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Not applicable

5.5 Significant changes in use or care of human subjects

Not applicable

5.6 Significant changes in use or care of vertebrate animals.

Not applicable

5.7 Significant changes in use of biohazards and/or select agents

Not applicable

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications.

A manuscript has been prepared for Nature Nanotechnology title "Recovery of Exosomes Containing Circulating Cell-Free RNA from Human Plasma Using High Conductance Dielectrophoresis", authored by Stuart Ibsen, Jennifer Wright, Sejung Kim, Seo-Yeon Ko, Jiye Ong, Sareh Manouchehri, Johnny Akers, Clark Chen and Michael J. Heller.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Some project results on exosome and ccf-RNA isolation by DEP have been presented at recent technical meetings, these included: **Fusion Conference Limited, Personalized Medicine,** Tucson, AZ, Sept. 30 , 2015; **SelectBio Conference NGS, SCA, MS & The Road to Diagnostics**, Del Mar, CA, Sept. 29, 2015; **San Diego BRAIN Consortium Symposium: Neurotechnology Research in San Diego** , La Jolla, CA, Sept. 19, 2015; **CNAPS 2015**, Berlin, Germany, Sept. 11, 2015; **CHI Next Generation Dx Summit**, Washington DC, August 18, 2015; **CHI Molecular Medicine TriConference,** San Francisco, March 10, 2016; and **CHI Next Generation Dx Summit**, Washington DC, August 23, 2016

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

New DEP MicroArray (Chip) and system protocols were described in the above Accomplishments section of the report.

Inventions, patent applications, and/or licenses

An invention disclosure titled “Electrokinetic Devices and Methods for Sample to Answer Molecular Diagnostics for Exosomes, RNA, DNA, Protein and other Biomarkers“ will be sent under separate cover to our Science Officer, Dr. Anthony Pacifico, and our Neurotrauma Research Portfolio Manager, Dr. Alicia Tamara Crowder (U.S. Army Medical Research and Materiel Command Combat Casualty Care Research Program).

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORTING ORGANIZATIONS

- **Individuals that worked on the project**

Personnel	**Role**	**Percent Effort**
Dr. Michael J. Heller	Project PI – Provide overall management and direction for the TBI project.	0.25 cal mos
Dr. Clark Chen	Project Co-PI – Prepare project IRB and provide TBI samples. expertise on brain-related RNA.	0.25 cal mos
Dr. Jennifer (Marciniak) Wright	Post Doc – Main role is to develop RT-PCR primers and protocols for testing RNA from blood and plasma.	50%/6 cal mos
Daniel Heineck	Graduate student research – DEP systems	13%/1.3 cal mos
Taeseok Oh	Graduate student researcher – Initiated work on on-chip	34%/3.4 cal mos

	fluorescent detection of RNA	
Sejung Kim	Post Doc researcher- Carried out general work for TBI project and SEM work	23%/2.3 cal mos
Dr. James Wurzbach	Co-PI Raytheon- The UCSD-Raytheon sub-contract has been completed.	0%

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

CoPI Dr. James Wurzbach (Raytheon) has been replaced by Dr. Adriane Stebbins (Raytheon)

- **What other organizations were involved as partners?**

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**

Nothing to report

9. APPENDICES (Raytheon Report and Quad Chart)

MRMC/UCSD Rapid Isolation and Detection for RNA Biomarkers for TBI

Annual Technical Report (Redacted Version)

Technical and Cost Report embedded in Annual Report

Subaward Number: 56441140

US Army MRMC Prime Award Number: W81XWH-14-2-0192

Issued 10/21/2016

Quarterly Technical Report Covering the Period 10/01/2015 – 10/01/2016

(Financial Information through 10/02/2016

1. CURRENT ANNUAL TECHNICAL REPORT 10/01/15 – 10/01/16

(Current Annual Technical Report is redacted due to potential export control and patentability. Upon request, a full report can be provided directly from Raytheon to Army.)

An underlying premise of the instant program (Rapid Isolation and Detection for RNA Biomarkers for TBI Diagnostics) is that certain variants of RNA and DNA can be sensitive and specific markers for TBI. Customarily, the TBI community invokes the Receiver Operating Characteristic (ROC) curve as the mechanism for quantifying sensitivity and selectivity, and therefore, the efficacy of a given nucleic acid variant as a discriminating marker for TBI. However, a frequent constraint is that the quantity of RNA and/or DNA is far below the limit of detection for most sensors of choice, e.g., fluorescence. Therefore, the scarce amount of RNA/DNA sample is amplified by use of the Polymerase Chain Reaction (PCR). Two significant consequences arise from invoking PCR.

1. PCR amplifies the original amount of RNA/DNA, but PCR does not provide a direct, quantitative measure of the original RNA/DNA that was present before amplification.
2. Appending PCR to the analysis adds at least 30 min, and more likely an hour, to the determination of TBI.

In regard to the first consequence, most workers address the qualitative result of PCR by choosing the threshold crossover cycle (C_T) as a surrogate measure of the RNA/DNA originally in the sample. The issue with using C_T in the calculation of ROC curves is that C_T is a logarithmic representation. The quantity of RNA/DNA doubles with each PCR cycle, yielding exponential growth. Another issue is that the cycles are discrete rather than continuous. Yet, historically, the ROC was derived by radar engineers using [nearly] continuous, linear data. Therefore, the impact of inserting exponential PCR cycle data into ROC curve calculation was examined in our Annual Report with an analysis of "Exponential Quantization Effects on Decision Thresholds."

In the quarter just completed, we continued our analysis with the incorporation of mathematical models to assist in characterizing and quantifying PCR curves. We note that workers employing conventional methods of PCR analysis attempt to interpolate a fractional value of C_T by applying linear regression to a log/log plot of fluorescence vs. cycle in the immediate vicinity of the threshold crossing. The regression analysis is limited to, literally, just a few points (three or perhaps four) to remain within a suitably linear region around the threshold crossover. Therefore, the accuracy of the interpolation result depends on a fit involving just three or four points. Our exploration with models seeks to improve the quantification by including as many points as practical from the threshold till the end of the PCR run.

It is understood that the proof-of-principle for the present program does not require a quantitative result. A qualitative assessment of the presence or absence of specific RNA/DNA variants as markers for TBI will suffice. But the analysis documented in the Annual Report, and continued in this quarter, anticipates subsequent program phases in which prediction of patient outcome will emerge as a high priority. At that point, it is expected that quantitative determinations will become increasingly relevant.

Our specific approach to quantification has not been observed in our review of PCR literature to date. It is, therefore, being considered for a patent as an element of a larger portfolio of signal processing algorithms to support TBI detection and prediction of patient outcome. Also, to the extent that our Exponential Quantization Effects analysis reveals implications for radar and sonar processing based on log amp detectors, the material may, potentially, be export controlled. Therefore, the following summary has been redacted pending a patent decision and determination of export limitations.

Initial efforts for automatic decision and prognosis focused on single measurement thresholding, and by analogy with radar and sonar signal processing, the Receiver Operating Characteristic (ROC). The ROC is used routinely in relevant, modern literature. Attention has quickly changed to consideration of automatic optimal decision making in light a vector of measurements, each component of which is an indicator (blood marker) of injury depending on the level of its measurement. It is important to keep in mind that measurement data is corrupted by a variety of "noise" sources, and our investigation has initially treated the accumulated noise effects as a random variable having a Gaussian probability distribution. Further insight on the covariance of the measurements is expected with further analysis, and this will eventually have an impact on the value of treating the vector all at once versus the simpler approach of treating the components individually and then combining results (in a voting model, say). There is a rich literature in generic statistical analysis of random vectors, and as a starting point, we have referenced the book by Mardia, Kent, and Bibby, *Multivariate Analysis*. Quick mention of another relationship that ties Traumatic Brain Injury diagnosis and radar signal processing is via the analogy between Adaptive Beam Forming and Multivariate Analysis in Mardia, *et al*., when the combined vector of blood marker measurements is considered.

For further progress to be made, attention turns to exactly the nature of the raw data feeding the multivariate analysis processes. Among the many papers on quantitative real-time PCR, we have initially focused on those that suggest a specific mathematical function to model the growth in target molecules during PCR amplification. As an example only, we illustrate with reference to a paper by Scot G. Frank and Philip S. Bernard on "Profiling Breast Cancer Using

Real-Time Quantitative PCR", included in the book *Rapid Cycle Real-Time PCR – Methods and Applications* edited by Carl Wittwer, Meinhard Hahn, and Karen Kaul for Springer-Verlag, NY 2004. The fluorescence measurements made after each PCR cycle form a short (say 40-50 cycles) sequence of values that ideally fit to a function of the form,

$$f(C;X) = X(2^C - 2C + 1),$$

but due to the noise in the measurement system and the eventually saturation of the sensors and depletion of amplification agents, the data better fits a logistics function of the form,

$$g(C;L,k,C_0) = \frac{L}{1+e^{-k(C-C_0)}}.$$

This mathematical representation of measurements is illustrated in the figure where C is the integer-valued PCR cycle index. The variable X would be the amount of blood marker molecules initially in the sample, C_0, is the (possibly fractional) PCR cycle number when the fluorescence level achieves the halfway point to the saturation level, L, and finally k is the efficiency of the equipment and would ideally equal 1 (100%) so that f would closely match[1] g for moderate PCR cycle numbers. The problem of quantifying the value of X from the measurement of L, k, and C_0 is the subject of many papers. However, initial focus in applying PCR to TBI diagnosis and a treatment should be, in our opinion, the generation and validation of such a family of mathematical functions to model each component of the blood marker vector. If for example, the logistics function modeled well for TBI blood markers, we would want to establish (calibrate) normal values for the equipment that determines parameters L and k for each of the

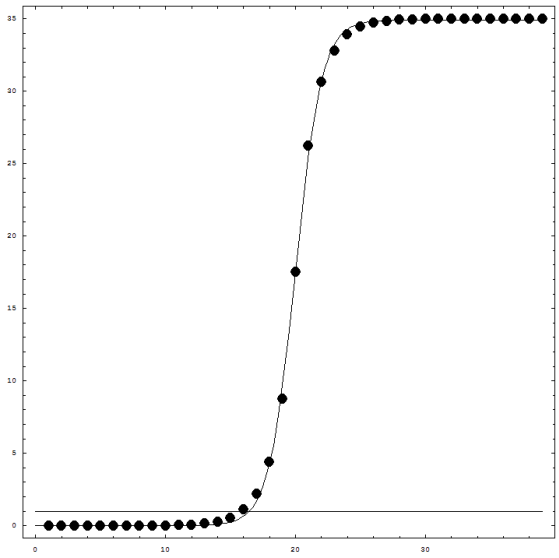


Figure illustrating Mathematical modeling of Fluorescence measurements during PCR cycling.

[1] Note that f(C, X) is a model for the number of molecules after PCR while g(C, L, k, C_0) is a model for fluorescence. Our sense of matching these models includes a scaling factor not shown.

vector components. The book by Wittwer, *et al.*, includes other papers illustrating PCR measurements where a logistics function model would not fit well.

In addition to the logistics model, a family of reasonably parameterized functions would fit PCR measurement data. For example, the logistics function satisfies a parameterized first order ordinary differential equation:

$$\frac{dg}{dC} = \frac{k}{L}\, g(C)(L - g(C)), and\ g(C_0) = \frac{L}{2}.$$

The behavior reported in some papers, after initial saturation, is for the fluorescence measurement to subsequently decay, and such behavior can be modeled after solutions to a parameterized family of second order differential equations, as illustrated in the figure when solving

$$a\frac{d^2g}{dC^2} + \frac{dg}{dC} = \frac{k}{L}\, g(C)\big(L - g(C)\big), and\ g(0) = b,\ \ g'(0) = c.$$

If this model worked well, two additional parameters would need to be identified for the normal population, here denoted a and c.

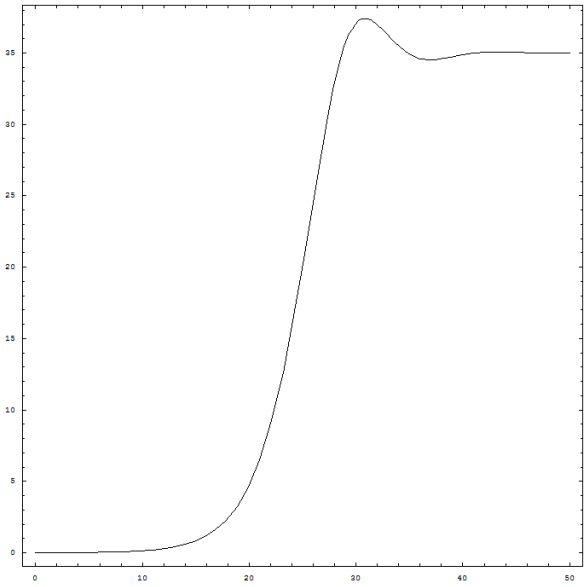


Figure illustrating alternative Mathematical modeling of Fluorescence measurements during PCR cycling.

Moving now to the second consequence listed above, we note that appending PCR to the DEP analysis will add 30-60 min to the determination of TBI. And while it is understood that this is still a dramatic improvement compared to legacy approaches, the additional time delay still encroaches upon military medics who seek a more immediate result for the diagnosis of injured warfighters. This has led Raytheon to investigate the feasibility of constructing a fluorescence detection apparatus capable of directly counting the scarce fluorophores that DEP isolates from a blood sample. The challenge is technically daunting. At the current limit of detection, DEP will successfully isolate some 50 target molecules in a given blood sample. Assuming all 50 target molecules are labelled with a fluorescent tag, a direct-counting system would have to detect just 50 fluorophores in the blood sample. If this level of sensitivity can be realized, then it would

obviate the PCR step by providing an immediate count of the target molecules in the sample, a significant step forward in the utility of the DEP technology.

To determine whether the 50-fluorophore detection is likely to be achievable, link budgets were calculated for a notional optical system. (A link budget is a serial calculation of the optical performance associated with each element in an optical chain to estimate the performance of the entire optical chain.) Two link budgets were assessed: one for a commercially available, high sensitivity CMOS camera; and another for a recently developed scientific camera (also commercial). The results indicated that the CMOS camera did not have the requisite sensitivity, but the research camera could conceivably succeed.

The sensitivity link budget estimate on the high sensitivity CMOS camera follows below.

detector [RGB]

wavelength [m]	frequency [Hz]	photon energy [J]	photon energy [eV]	detector sensitivity spec [Lux s]	frame rate / shutter speed [s]	detector sensitivity spec [Lux]	detector sensitivity spec [W/cm^2]	detector size [cm]	number of photon equivalent sensitivity during integration time	well depth [e]
5.50E-07	5.45E+14	3.61E-19	2.25E+00	2.80E-02	3.00E-02	9.33E-01	1.36E-07	5.86E-04	3.89E+03	3.00E+04

optics

front aperture diameter [cm]	pupil size [cm]	F number	system focal length [cm]	diff limited blur spot diameter [cm]	deviation form diffraction limit	actual blur spot diameter [cm]	blur spot area [cm^2]	entrance pupil area [cm^2]	optical transmission	sensitivity at aperture [Lux]	sensitivity at aperture [photons per second]	number of photon equivalent sensitivity during integration time	collection efficiency	number of photon equivalent sensitivity during integration time at sensor
5.08	1	1.5	1.5	2.01E-04	3	6.04E-04	2.86E-07	7.85E-01	8.00E-01	4.25E-07	1.35E+05	4.05E+03	2.22E-01	1.82E+04

Laser excitation

object area [cm^2]	laser power [W]	laser wavelength [cm]	number of fluorophore emitters	laser photon energy [J]	fluorophore decay fluorescence lifetime [s]	laser intensity at object [W/cm^2]	laser excitation rate [photons per second]	maximum "cycles" - based photon emission rate
1	0.005	0.00005	50	3.97E-19	1.00E-08	5.00E-03	1.26E+16	5.00E+09

•**Molar absorption coefficient: 2.5x10^5 [cm^{-1}M^{-1}] gives absorption cross-section of 4.15x10^{-16}[cm^2]**

$$\sigma_A[cm^2] = \frac{\varepsilon_A\left[\frac{liter}{cm*mol}\right]*1000\left[\frac{cm^3}{liter}\right]}{N_A\left[\frac{1}{mol}\right]}$$

absorption efficiency

Molar absorption x-section [M^{-1} cm^{-1}]	Molar absorption x-section [cm^2]	fluorescent tagged emitter layer thickness [cm]	fluorescent tagged emitter layer area [cm^2]	Number of emitters per unit volume [cm^{-3}]	linear absorption coefficient [cm^{-1}]	absorption efficiency	laser excitation rate [photons per second]	photons excited per integration time [s]	equivalent photons at sensor level
2.50E+05	4.15E-16	1.00E-02	1	5.00E+03	2.08E-12	2.08E-12	2.61E+04	7.83E+02	1.67E+02

Figure 1. High sensitivity CMOS camera link budget estimates

Starting with the sensitivity specification at the detector level of 0.028 Lux s, for 30 ms shutter speed/integration time, the detector level sensitivity is 0.933Lux. The conversion from Lux to intensity [W/cm^2] is 1.46 x 10^{-7}. For a nominal signal fluorophore emission wavelength of 550 nm, this corresponds to an equivalent sensitivity during integration time of ~ 4000 photons. It should be noted that a typical well depth for these CMOS imagers is ~ 30,000 electrons. Hence, although one could increase integration time to approach this photoelectron number accumulation, it is preferred to stay at a smaller fraction of the full well depth in order to maintain the desired dynamic range for consistent SNR.

For (notional) medium sized collection optics (5cm diameter) and a fast f-number (1.5), and given the solid angle-based estimated collection efficiency (assuming diffuse line emission from the sample object), the overall sensor system has an actual estimated photon equivalent sensitivity during integration time of ~18000 photons.

Assuming a notional case of 50 emitting fluorophores per 1 cm square object area, the laser excitation that would be required to achieve the needed emission rate commensurate with the CMOS sensor sensitivity is ~ 5mW. For "waveguide"– coupled excitation, and assuming a 100 micron thick waveguide base layer, the 5 mW laser intensity would corresponds to ~500 mW/cm^3 volumetric intensity (power density) – which is probably close to optimum before Joule heating starts affecting the interrogated sample. It is, of course, desired to limit the intensity of excitation at the fluorophore level, as there would invariably be heating of the biological molecules. The next assumption used is average absorption efficiency in the sample based on typical molar extinction coefficients. For a notional molar absorption coefficient of 2.5 x 10^5 $M^{-1}cm^{-1}$, the absorption efficiency for a sample containing 50 fluorescent tagged molecules is ~ 2 x 10^{-12} cm^{-1}. This results in a maximum photon excitation rate of ~2.6 x 10^4 photons per second, or ~800 excited photons per integration period – and ~ 170 equivalent photons collected at the sensor system level. For this notional sensor implementation and associated fairly fast collecting optics, the minimum fluorescent tags that can be seen is actually ~5000 rather than the target 50. Optimization might improve the situation, but for practical purposes, the CMOS camera does not seem likely to provide the needed sensitivity.

Turning now to the scientific camera, we analyze a similar link budget. It is important to note that the two sources of noise (read noise and dark current noise) will impact sensitivity depending on integration time. The read noise for this sensor is as low as 1.6 electrons at 100 frames per second (or 10 ms integration time). The dark current is smaller – 0.6 electrons per second per pixel; however, this will grow with longer integration times.

At the pixel level, this sensor is likely to achieve the target 50 photon level sensitivity detection performance. For notional SNR performance of ~10, the scientific camera can probably be sensitive to ~60 photons (50 photons would be an SNR of ~8). The equivalent photons at the sensor level (taking all transmission losses, transfer losses , and efficiency into account) translates into a laser power requirement of ~ 10 mW, which is reasonable for maintaining the integrity of the bio samples, and yet achieving the photon emission rates required for the desired level of sensitivity.

Cost Report – Raytheon Proprietary Information

Customer	UCSD
Contract #	56441140
Program Name	TBI
Month Reporting	Sep-16

MTD Hrs	MTD charges	ITD Hours	ITD Charges
39.6	$ 7,444.12	1013.5	$ 185,815.83

PRIOR ITD Hours	PRIOR ITD Charges
973.9	$ 178,371.71

Rapid Isolation and Detection for RNA Biomarkers for TBI Diagnostics

Log Number 13212004 Year 2 – Annual Report Sept 30, 2016

Award Number W81XWH-14-2-0192

PI: Michael J. Heller **Org:** University of California San Diego **Award Amount:** $ $1,871,874.00

Study/Product Aim(s) - Most of the Year One and Year Two goals not related to final IRB approval and use of TBI patient samples have been accomplished. While our milestone for obtaining the UCSD IRB was achieved (May 2015), the IRB was not approved by HRPO-ORP-USAMRMC until June 2016. Nevertheless, we successfully demonstrated the basic capability of our DEP technology for the isolation of glioblastoma exosomes (containing cf-RNA) from 50 mL of undiluted plasma in 20-30 minutes. We then showed tri-color fluorescent detection of the isolated exosomes (red fluorescence), cf-RNA (green fluorescence) and cf-DNA (blue fluorescence). Glioblastoma-specific mRNAs for EGFRvIII and β-actin biomarkers were identified by RT-PCR carried out on m-RNA from exosomes. We have also developed a novel on-chip method to carryout immunofluorescence analysis of brain specific exosomal protein biomarkers.

Approach - Overall project goal is to demonstrate that high conductance dielectrophoretic (HC-DEP) devices & technology can be used to carry out rapid isolation and detection of TBI related ccf-RNA (mRNA, miRNA) exosomes and other biomarkers from blood, plasma & serum.

Activities Year 2 (10/14-12/15)	**Q-5**	**Q-6**	**Q-7**	**Q-8**
First Specific Aim & Tasks:	Milestone 1			
Second & Third Specific Aim & Tasks: *"Completed with glioblastoma samples"*	Milestone 2 & 5			
Completed IRB and UCSD approval	Milestone 3			
Fourth Specific Aim & Tasks (Raytheon)	Milestone 4			
Fifth Specific Aims & Tasks (No IRB)				
Estimated Budget ($K)	**$900**	**$990**	**$1,098**	**$1,267**

Updated: (Sept 30, 2016 – This 8th Quarter/Year 2)

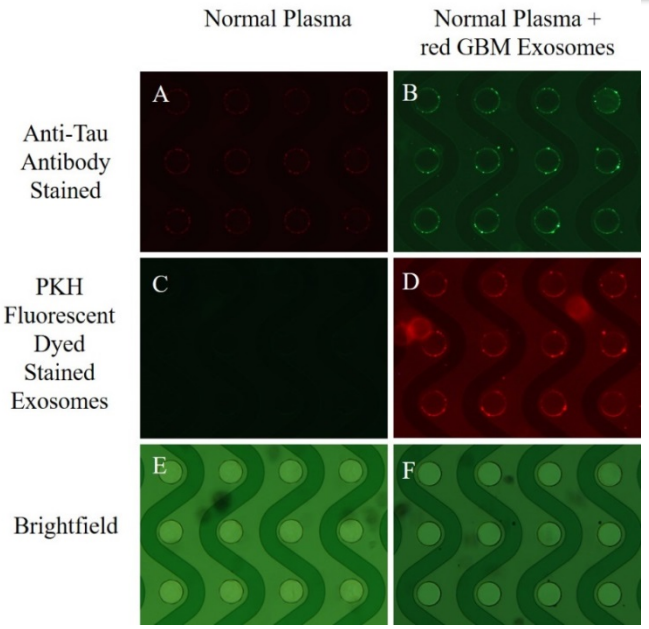


Figure 1 - DEP isolates PKH (red)-dyed glioblastoma exosomes spiked into normal plasma and identified for the brain-specific marker Tau. *Exosomes purified from cultured cell supernatants and stained with the non-specific membrane stain PKH (red) were spiked into normal human plasma. Following isolation onto the DEP chip, exosomes were permeabilized with saponin, and positively labeled with antibody selective for Tau protein. (A and B) Anti-tau antibodies were visualized with Alexa Fluor 488-conjugated secondary antibody (green). (C and D) Fluorescence image of same electrodes, positive for PKH-labeled exosomes (red). (E and F) Electrodes corresponding to panels directly above, viewed with bright field.*

Year 2 Goals/Milestones - Continue to *carry out and optimize RT and qPCR reactions, and test PCR primers on RNA spiked samples, and glioblastoma/exosome samples completed.* **Second & Third Specific Aim & Tasks/Milestones** completed using *glioblastoma exosome samples, until TBI patient samples become available (now available)*

Comments/Challenges/Issues/Concerns - Since our project IRB was not approved by HROP until June 2016, we were not been able to carry out tasks requiring TBI patient samples (TBI samples now available)

Projected Expenditure to date ~$1,267,586.93.

Actual Expenditure: On track considering EWOF and IRB final approval